Membrane integrity of Bacillus stearothermophilus

Gregg Allen Mosley

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MEMBRANE INTEGRITY OF *Bacillus stearothermophilus*

By

Gregg A. Mosley

B.A., University of Montana, 1969

Presented in partial fulfillment of the requirements for the degree of

Master of Science

UNIVERSITY OF MONTANA

1972

Approved by:

[Signatures]

Chairman, Board of Examiners

[Signature]

Dean, Graduate School

[Signature]

Date:

Dec 4, 1972
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<td>Explanation</td>
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</tr>
<tr>
<td>&quot;Bis&quot; acrylamide</td>
<td>N, N'-Methylenebisacrylamide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DPG</td>
<td>Diphosphatidyl Glycerol</td>
</tr>
<tr>
<td>$K_m$</td>
<td>Michaelis Constant</td>
</tr>
<tr>
<td>K-PO$_4$ buffer</td>
<td>0.001M Potassium Phosphate Buffer (pH 6.8)</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethyl Maleimide</td>
</tr>
<tr>
<td>ONP</td>
<td>Ortho-Nitrophenol</td>
</tr>
<tr>
<td>ONPG</td>
<td>Ortho-Nitrophenyl Galactoside</td>
</tr>
<tr>
<td>PG</td>
<td>Phosphatidyl Glycerol</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic Acid</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N,N-Tetramethyl Ethylene Diamine</td>
</tr>
<tr>
<td>Tm</td>
<td>Thermal Melting Point</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris (hydroxymethyl) Aminomethane·HCl</td>
</tr>
<tr>
<td>TSA</td>
<td>Tryptcase Soy Agar</td>
</tr>
<tr>
<td>TYE</td>
<td>Broth Culture Medium of 2% Tryptcase + 1% Yeast Extract</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>Maximum Velocity</td>
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</table>
CHAPTER I

INTRODUCTION

Thermophilic bacteria have been the subjects of numerous research efforts since their occurrence was first recorded by Miquel in 1879. The usual object of such research has been to elucidate the basis for their thermophilic nature. There have been various explanations for the ability of these organisms to proliferate at temperatures too high to allow even the survival of the more ubiquitous mesophilic organisms.

One of the early explanations for thermophily centered around the concept of rapid resynthesis of denatured protein by thermophiles. The observation that mesophile and mammalian protein often coagulated at temperatures optimal for thermophilic growth led Allen (1, 2) to propose that rapid resynthesis of denatured protein could enable organisms to survive at higher temperatures. Allen (2) based her conclusion on the observation that the thermophile, Bacillus stearothermophilus, survived no better than the mesophile, Escherichia coli, in a non-nutritious sodium bicarbonate buffer (pH 8.0) at 55°C. Her evidence indicated that non-growing thermophilic populations were no more heat-stable than mesophilic ones.

Recently, Bubela and Holdsworth (9, 10) have reported that B. stearothermophilus has a greater rate of protein and nucleic acid (mRNA) turnover than E. coli when both are growing near their optimal temperatures. The rates were also higher for the thermophiles when both...
were grown at 40°C. The authors did not, however, claim that this was the mechanism for thermophily, but only pointed out that the capacity for replacing protein and nucleic acids was greater in the thermophile than in the mesophile.

Another hypothesis which gained widespread support is that the molecular components of the thermophiles are innately more heat-stable than those from mesophiles. As Koffler (25) has noted, this could be due to an inherent property of the molecules or could be caused by protective factors. Heat-stable enzymes and proteins in thermophiles have been recognized since 1949 (40). Many of these have been purified and characterized physically (3, 4, 12, 24, 33, 36, 37, 41, 42, 43, 60). Evidence supporting the hypothesis of the innate heat stability of thermophilic enzymes was obtained by Manning and Campbell in studies of the alpha-amyrase of B. stearothermophilus (11, 33). The amino acid composition of this heat-stable alpha-amyrase was different from that of alpha-amyrase of mesophilic organisms: tryptophan was absent, there was a lower amount of tyrosine, and there were higher amounts of aspartic acid, glutamic acid and proline (11). Furthermore, optical rotation studies indicated that the enzyme molecule was largely unfolded and well hydrated, which may account for the thermostability of the amylase (33). However, an unfolded structure is not universal to thermostable proteins, as Koffler has noted with flagellin (24). He found that the purified flagellin from B. stearo-
thermophilus was more resistant to thermal denaturation than were purified flagellins from several mesophilic bacterial species. It was observed that flagellin obtained from B. stearothermophilus also was
more resistant to denaturation by urea and acetamide, from which Kof­
fler concluded that the thermostability was due to increased hydrogen bond­ing within the thermophile flagellin. In addition, Koffler showed that the majority of the cytoplasmic proteins of thermophiles are much more resistant to heat denaturation (coagulation at high temperatures) than similar proteins from several mesophiles.

Comparisons between mesophiles and thermophiles has by no means been limited to their consituent proteins. Increased thermosta­bility has been reported for ribosomes and rRNA from thermophiles (50). Pace and Campbell (45) have reported that the thermal melting point \( T_m \) values for ribosomes from a number of organisms correlated very well with their maximum growth temperatures. Saunders and Campbell (50) also have reported that the amino acid composition of the thermophile ribosomal protein was not unusual, but that it did contain slightly more half-cystine residues than did mesophile ribosomal pro­tein. They concluded that the half-cystine residues could have formed disulfide linkages within the protein, which could confer increased thermostability, but that the number of potential disulfide linkages was insufficient to account quantitatively for the increase in thermo­stability.

Stenesh et al. (57) have compared the DNA and mRNA from *E. coli* and *B. stearothermophilus*. Their results indicate a higher \( \frac{G+C}{A+T} \) ratio for the thermophile DNA (1.13) compared to the mesophile DNA (0.82). The \( G+C \) content of the RNA from the thermophile also was higher than that from the mesophile. It should be noted, however, that the \( \frac{G+C}{A+T} \) ratios vary considerably among mesophiles (34),

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and Stenesh concluded, "It is thus apparent that in the overall physical parameters investigated here, the DNA's of the mesophiles did not differ significantly from the DNA's of the thermophiles."

There have been several studies (15, 59, 65) reported on the composition of cell walls of *B. stearothermophilus*. Sutow and Welker (58) reported that with increasing temperatures of growth, the cell wall component peptidoglycan increased and teichoic acid decreased when expressed as percent dry weight of whole cells. The conclusion reached by different researchers (15, 65) is that the cell walls from the thermophile, *B. stearothermophilus*, is very similar to cell walls from three mesophilic members of the genus *Bacillus*.

The membrane and constituent lipids also were components of thermophiles considered worthy of characterization. Gaughran (17) found that with increasing temperature of growth, bacteria possessed decreased amounts of unsaturated fatty acids. Since Gaughran's early work, numerous groups have investigated the lipids and membranes of thermophiles and the effect of growth temperature on the lipid composition of the cell (7, 8, 10, 12, 13, 31, 35, 44, 46, 47, 52, 66). The results indicate that the effects of increasing temperature of growth on the lipid composition are: (1) increased amount of saturated fatty acids (16, 34, 44, 47); (ii) increasing fatty acid chain lengths (35, 44, 47); (iii) a decrease in cellular neutral lipid content (44). The resulting effects of (i) and (ii) are membranes with increased melting points and, therefore, thermostability (17, 35). Both Brock (8) and Friedman (16) have implied that the membrane and its role in organization of cellular metabolism may be the most critical component.
In the thermostability of thermophilic organisms. Brock (8) has cited three lines of evidence in support of this contention: (i) membrane lipid and fatty acid composition; (ii) thermal death follows single order kinetics indicative of a single hit prediction rather than a multiple hit; (iii) Brock's observations that maximum growth temperature decreases with increasing amounts and complexity of membrane components. He observed increasing maximum growth temperatures for eukaryotes, photosynthetic procaryotes, and non-photosynthetic procaryotes in that order. Thus, disorganization of multi-enzyme systems or decrease in thermostability of enzymes upon dissociation from the membrane may affect the decrease in thermal viability of an organism. Results of Bubela and Holdsworth (10) indicate that the amino-acyl activating enzymes in B. stearothermophilus are membrane-bound systems which can be solubilized without significant loss in activity. The solubilized enzymes, however, are much more heat-labile than the membrane-bound forms.

Recently, Ljunger (30) reported on conditions affecting the thermal death rates of B. stearothermophilus. He found that the thermophile rapidly lost viability when incubated in Tris (tris (hydroxymethyl) aminomethane) buffer at 65°C. If, however, calcium and glucose (or one of several other sugars) were added to the buffer, the survival of the cells was dramatically prolonged. Ljunger contended that this effect was due to the protection of cellular proteins from heat denaturation by the calcium ions. This effect was attributed to active transport of calcium and maintenance of a high intracellular concentration of the ion, although he gave no experimental evidence.
for such active transport. He cited the work of Manning and Campbell with alpha-amylase as support for this contention. This enzyme had been shown to require calcium for activity and this has been interpreted by Ljunger as implicating calcium as a stabilizing factor for the enzyme. The results from his viability studies indicated that the effect was specific for calcium, and that other divalent cations could not replace it. The requirement for inorganic stabilizing factors, however, is not universal, since other heat-stable enzymes, such as aldolase (60), glucose-6-phosphate isomerase (43), and glyceraldehyde-3-phosphate dehydrogenase (3), have lacked such metal ion requirements. In fact, Thompson (60) was careful to note that the aldolase was a metal ion-independent thermostable enzyme. His conclusion, therefore, did not seem justified in the light of this previous research and because active transport of calcium had not been demonstrated.

Previous work in this laboratory indicated that the conclusions of Brock (8) and Friedman (16) are correct, i.e., the membrane and the cellular organization associated with it represent the most thermosensitive component of thermophilic organisms. Considerable data has been collected in this laboratory on the lipid composition and turnover in _B. stearothermophilus_. This organism appears to provide an ideal system for studying the effect of lipid composition on membrane integrity, cellular organization, and thermal viability.

Originally, this thesis was intended to report the results from an intensive study on the alpha- and beta-galactosidases and the galactoside transport system in _B. stearothermophilus_. Preliminary studies, however, indicated that membrane stability was not maintained
under galactosidase assay conditions. The results reported by Ljunger, therefore, were of special interest because of these observations. Furthermore, Dr. Card (private communication) had adduced evidence that the membrane phospholipid composition of \textit{B. stearothermophilus} rapidly changed when cells were deprived of oxygen and held at 60°C. These cells rapidly converted phosphatidyl glycerol (PC) to diphosphatidyl glycerol (DPG) without any loss in viability. He also had noted that the high DPG cells formed much more stable protoplasts upon treatment with lysozyme than did the low DPG cells.

The calcium effect reported by Ljunger and our own evidence led to the hypothesis that calcium ions stabilized the membrane and resulted in increased thermostability. Both calcium (possibly other divalent cations as well) and high DPG should stabilize the membrane against leakage of intracellular components and disorganization of multi-enzyme systems associated with the membrane as well as increase the thermal viability of the organism. The data subsequently presented in this thesis strongly support this hypothesis. Furthermore, these data, in conjunction with studies reported by other researchers, suggest that the effect of calcium is a direct one on the membrane, and that interaction with the membrane lipids is implicated by the work of others (38, 49, 58) with lipid bilayers.

Since the galactosidases proved so important as indicators of cell lysis, and because they previously had not been reported in this organism, it seemed necessary to include data on some of their physical parameters.
CHAPTER II

MATERIALS AND METHODS

ORGANISM AND CULTURE MAINTENANCE

The organism used in these studies was Bacillus stearothermophilus NCA 2184. This was part of the stock culture maintained by Dr. G. L. Card. Cells were grown and refrigerated on slants of Trypticase Soy Agar (TSA). Weekly cultures were those inoculated from TSA slants into 2% Trypticase + 1% Yeast Extract (TYE) and grown at 60°C in a New Brunswick shaker incubator to early log phase (O.D. at 600 nm 0.15 to 0.30). This cell suspension was refrigerated and was used as inoculum for subsequent experiments during the week.

CHEMICALS AND CULTURE MEDIA

Alpha- and beta-ONPG (ortho-nitophenyl galactoside), lysozyme, NEM (N-ethyl maleimide), and Tris (tris (hydroxymethyl) aminomethane) were obtained from Sigma Chemical Company, St. Louis, Missouri. Acrylamide, TEMED (N,N,N,N-tetramethyl ethylene diamine), and "bis" acrylamide (N,N'-methylenebisacrylamide) were obtained from Eastman Organic Chemicals, Rochester, New York. ONP (ortho-nitrophenol) and D (-) Ribose were obtained from J. T. Baker Chemical Company, Phillipsburgh, New Jersey. Difco of Detroit, Michigan supplied Yeast Extract and TC Vitamins Eagle 100x. Trypticase and Trypticase Soy Agar were received from BBL, Cockeysville, Maryland. Nutritional Biochemical Corporation of Cleveland, Ohio supplied calf thymus DNA. Riboflavin was obtained...
from the Matheson Company, Incorporated, Norwood, Ohio. All other chemicals were reagent grade obtained from various commercial sources. All preparations requiring sterilization were autoclaved at 15 psi at 121°C for 15 minutes unless otherwise specified.

ENZYME CHARACTERIZATION

Enzyme Preparation

Preparations for characterization were centrifuged, dialyzed cell lysates unless otherwise specified. These were obtained by inoculating 500-ml batches of TYE in 2 liter baffled flasks and allowing growth to late-log or stationary phase (O.D. at 600 nm 0.80 to 1.0). Cultures were poured into 250 ml plastic centrifuge tubes, centrifuged at 1,400 x g in an International centrifuge, size 2, model V, for 20 minutes. Cells were washed twice in 0.1M potassium phosphate buffer (pH 7.0) and collected by centrifugation in 50 ml plastic centrifuge tubes at 12,000 x g for 15 minutes in a Sorvall RC2-B centrifuge.

Cells were lysed by resuspending them in 30.0 ml distilled water + lysozyme (100 µg/ml). After 20 to 30 minutes at room temperature, suspensions were briefly sonicated and subsequently centrifuged at 48,000 x g for 30 minutes in the RC2-B centrifuge. Supernatant fluid was removed and dialyzed in the cold against 20 volumes of 0.001M potassium phosphate buffer (pH 7.0) for 2 days with fresh buffer changes every 6 to 8 hours.

The assay procedure was a modification of the method described by Egan and Morse (14). One-half ml amounts of the lysates were added to 3.5 ml preheated 0.1M potassium phosphate buffer, and after 5 minutes
1.0 ml of alpha- or beta-ortho-nitrophenyl galactoside (ONPG) was added, giving the final concentration indicated in the legends. The final volume of the assay mixture was 5.0 ml, and after the desired time (7.5 to 20.0 minutes, depending on the enzyme concentration of the preparation) 2.0 ml of cold 0.25M Na₂CO₃ were added and the tube immersed in an ice bath. Controls were enzymes preparations without the addition of substrate. Unhydrolyzed substrate was checked for absorbance by comparing one control without addition of enzyme to the other control. Enzyme activity expressed was u moles ortho-nitrophenol (ONP) released per minute in the assay system. Ortho-nitrophenol was quantified by comparing the absorbance at 420 nm in 19.0 mm cuvettes on a Coleman II Junior spectrophotometer to a standard curve prepared with commercial ONP.

Determination of Non-Inducibility of Alpha- and Beta-Galactosidase

The defined medium prepared (Table 1) was similar to that described by Welker and Campbell (62) for B. stearothermophilus 1503-4; any changes are noted with an asterisk. Salts in Group 1 were sterilized by autoclaving at 15 lbs. pressure and 121°C for 15 minutes in a 10x concentrated solution. All other chemicals were added prior to inoculation. Those in Group II were dissolved in an appropriate amount of distilled water and were filter-sterilized. Amino acids were filter-sterilized in 10x concentrations. Carbohydrates were filter-sterilized as 20% solutions. Vitamins were commercial preparations of TC Vitamins Eagle 100x, the contents of which are listed in Table 2.
Table 1. Defined Medium for *B. stearothermophilus*

<table>
<thead>
<tr>
<th>Material Classification</th>
<th>Compound</th>
<th>mg/l</th>
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<tbody>
<tr>
<td></td>
<td>Group I</td>
<td></td>
</tr>
<tr>
<td></td>
<td>KH$_2$PO$_4$</td>
<td>1000.0</td>
</tr>
<tr>
<td></td>
<td>K$_2$HPO$_4$</td>
<td>2500.0</td>
</tr>
<tr>
<td></td>
<td>NH$_4$Cl</td>
<td>1000.0</td>
</tr>
<tr>
<td></td>
<td>NaCl</td>
<td>1000.0</td>
</tr>
<tr>
<td></td>
<td>Potassium acetate</td>
<td>500.0</td>
</tr>
<tr>
<td></td>
<td>Group II</td>
<td></td>
</tr>
<tr>
<td></td>
<td>*FeCl$_3$ · 6H$_2$O</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>*MgCl$_2$ · 6H$_2$O</td>
<td>25.0</td>
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<tr>
<td></td>
<td>*CaCl$_2$ · H$_2$O</td>
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<tr>
<td></td>
<td>Amino Acids</td>
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</tr>
<tr>
<td></td>
<td>L-Arginine</td>
<td>105.0</td>
</tr>
<tr>
<td></td>
<td>DL-Methionine</td>
<td>60.0</td>
</tr>
<tr>
<td></td>
<td>DL-Valine</td>
<td>144.0</td>
</tr>
<tr>
<td></td>
<td>Vitamins</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TC Vitamins Eagle 100x</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Carbohydrates</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glycerol, Lactose, Galactose, or Melibose</td>
<td>10,000.0</td>
</tr>
</tbody>
</table>

Table 2. Contents of TC Vitamins Eagle 100x

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Ca-Pantothenate</td>
<td>100.0</td>
</tr>
<tr>
<td>Choline Chloride</td>
<td>100.0</td>
</tr>
<tr>
<td>Folic Acid</td>
<td>100.0</td>
</tr>
<tr>
<td>i-Inositol</td>
<td>200.0</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>100.0</td>
</tr>
<tr>
<td>Pyridoxal HCl</td>
<td>100.0</td>
</tr>
<tr>
<td>Riboflavine</td>
<td>10.0</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>100.0</td>
</tr>
</tbody>
</table>
Cells were grown in 50 ml TYE in a 250 ml baffled, side-arm flask to an O.D. of 0.48. Ten ml were removed, centrifuged, and washed twice in minimal salts plus amino acids. The cells were then resuspended in 5.0 ml of the minimal salts plus amino acids. One-ml amounts of this cell suspension were used as inoculum for 50.0 ml of the minimal growth medium. After incubation for 4.5 hours at 60°C on the rotary shaker incubator (O.D. at 600 nm 0.15 to 0.17), the cells were centrifuged and washed twice in distilled water. Twenty ml of the mid-log TYE cells were treated similarly. Cells were suspended in 5.0 ml distilled water, 2.0 ml were removed, and dry weight was determined. Lysozyme (300 ugm) was added to the remaining 3.0 ml, and after 20 minutes the suspension was vigorously pipetted to complete lysis. Samples were then assayed for galactosidase activities.

Electrophoresis

Polyacrylamide procedure used was basically the anionic system described in a technical publication (22). Buffers and gels were made according to the scheme presented in Table 3.

Gels were prepared by mixing the precooled components in the cold according to the volume ratios indicated. Gel columns were prepared in glass tubing 95 mm x 7.0 mm (inside diameter). Resolving columns of 47 mm were made, and polymerization achieved by allowing the solution to come to room temperature. Stacking gel columns of 30 mm were layered over the resolving gel columns and polymerized using a table fluorescent lamp. A 0.5 ml enzyme preparation, as a 30% sucrose solution, was layered over the stacking gel and another stacking gel column of 10 mm was layered over the sample and polymerized.
Electrophoresis was carried out with a Buchler Model No. 3014 A as a power source. Amperage applied was 3 ma per tube for the first 5 minutes, 5 ma per tube for the next 15 minutes, and then 7 ma per tube for the remaining hour. Columns were removed from the tubes and the resolving gel cut into sections at 2.0, 2.5, 3.0, 3.5, and 4.0 cm from the gel interface. Each section was halved and crushed extensively in the assay buffer (0.1M potassium phosphate, pH 5.8) and one half was assayed for alpha-galactosidase and the other for beta-galactosidase. To allow for diffusion of enzymes out of the gel, these solutions remained at room temperature for 30 minutes and were incubated for 15 minutes at 60°C before addition of alpha- or beta-ONPG in 2 mM and 5 mM final concentrations, respectively. Gel sections from columns to which sample had not been applied plus substrate were the controls.
<table>
<thead>
<tr>
<th>Components</th>
<th>Volume Ratios</th>
<th>Compound</th>
<th>Amount/100 ml</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Resolving Gel</strong></td>
<td>1</td>
<td>Acrylamide</td>
<td>30.0 gm</td>
<td>——</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&quot;bis&quot; acrylamide</td>
<td>1.0 gm</td>
<td>——</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1M HCl</td>
<td>24.0 ml</td>
<td>8.9 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tris</td>
<td>18.15 gm</td>
<td>——</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TEMED</td>
<td>0.4 ml</td>
<td>——</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>(NH₄)₂S₂O₈</td>
<td>0.4 gm</td>
<td>——</td>
</tr>
<tr>
<td><strong>Stacking Gel</strong></td>
<td>2</td>
<td>Acrylamide</td>
<td>5.0 gm</td>
<td>——</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&quot;bis&quot; acrylamide</td>
<td>5.0 gm</td>
<td>——</td>
</tr>
<tr>
<td>1</td>
<td>1M HPO₄</td>
<td>12.0 ml</td>
<td>7.2 ± 0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tris</td>
<td>2.88 gm</td>
<td>——</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TEMED</td>
<td>0.1 ml</td>
<td>——</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Sucrose</td>
<td>20.0 gm</td>
<td>——</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Riboflavin</td>
<td>0.005 gm</td>
<td>——</td>
<td></td>
</tr>
<tr>
<td><strong>Upper Buffer</strong></td>
<td></td>
<td>Tris</td>
<td>0.632 gm</td>
<td>8.9 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Glycine</td>
<td>0.394 gm</td>
<td>——</td>
<td></td>
</tr>
<tr>
<td><strong>Lower Buffer</strong></td>
<td>Tris</td>
<td>1.21 gm</td>
<td>8.1 ± 0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1M HCl</td>
<td>5.0 ml</td>
<td>——</td>
<td></td>
</tr>
</tbody>
</table>
MEMBRANE STUDY

Growth and Harvest Conditions

*Bacillus stearothermophilus* was grown in TYE on the rotary shaker incubator at 60°C. Cells used for most experiments (exceptions are noted) were mid-log phase cells. These were obtained by growing the cells in a small volume of medium to mid-log phase (O.D. at 600 nm 0.30 to 0.45) and transferring them to a baffled, 2 liter culture flask containing 500 ml of preheated medium. Cultures were removed from the incubator, immediately poured over ice, harvested by centrifugation, and washed twice in cold 0.001M potassium phosphate buffer (pH 6.8).

Cell populations referred to as "anaerobic" were poured from the growth flask (mid-log phase cells) into an Erlynmeyer flask of just enough capacity for the culture fluid. At mid-log cell densities, dissolved oxygen is rapidly utilized and conditions become anaerobic. Cells were then harvested as described above. Dr. Card (personal communication) has indicated that no loss of viability occurs under such conditions for up to 30 minutes. Also, conversion of PG to DPG occurs, while synthesis of PG essentially stops. In subsequent discussion, "low DPG cells" will refer to those harvested at mid-log phase, and "high DPG cells" to those held anaerobically before harvesting.

Survival Studies

Cells were grown at 60°C in 50 ml TYE in a 250 ml baffled, side-arm flask on the rotary shaker incubator. At mid-log phase, samples were removed and immediately diluted $10^{-4}$ in 5.0 or 10.0 ml pre-
heated 0.01M Tris buffer (pH 7.2 at 25°C) with additions as indicated, or cultures were held "anaerobically" before such dilution. Samples were then removed at times indicated, plated neat, or serial dilutions of $10^{-1}$ were made as necessary in 0.1% trypticase. Plates were made in triplicate on TSA, incubated for 16 to 18 hours at 55°C, and counted with the aid of a New Brunswick colony counter.

**Protein Determination**

The twice-washed pellets described in the "Growth and Harvest Conditions" section were resuspended in cold buffers (usually 30 to 60 ml), divided into 2.5, 3.0, or 5.0 ml aliquots, and heated in a 60°C water bath. At indicated times, heated cell suspensions immediately were immersed in an ice bath for 2 minutes, then centrifuged at maximum speed in an International clinical centrifuge for 20 minutes at 4°C, and the supernatant fluids were removed carefully. Supernatant samples were frozen and held at -20°C if not used immediately. Protein was determined by the method of Lowry et al. (32). The standard was bovine serum albumin.

**Galactosidase Assay**

Whole cells to be tested were heated, transferred to an ice bath, and held no more than 2 hours until tested. Assays consisted of 0.5 ml amounts for whole cells, or 0.5 or 1.0 ml amounts for recently collected supernatant fluids in 0.001M potassium phosphate buffer (pH 6.8 or 5.8), plus 5 mM substrate alpha- or beta-ONPG in a final volume of 5.0 ml. Total activity was determined by adding lysozyme to whole cells (100 ugm/ml final concentration) and briefly sonicating.
after protoplast formation was complete. Whole cell assays also contained CaCl₂ (0.0125M) in the buffer for stabilization, and were centrifuged before checking the O.D. at 420 nm.

Release of Nucleotides

Supernatant fluids were tested for release of nucleotides by two methods: (1) Samples were tested for RNA content by the orcinol reaction of Dische (5) as described in Methods in Enzymology; samples of 0.3 or 0.5 ml amounts were diluted to 1.0 ml for testing and absorbance was compared to a standard curve prepared with ribose at concentrations ranging from 2 to 50 ugm/ml ribose: and (ii) samples were divided into two equal fractions, one being diluted 1:2 with 0.001M potassium phosphate buffer (pH 6.8) for determination of total nucleotides, while the other was diluted with buffer plus 14% trichloroacetic acid (TCA) for determination of the soluble nucleotides. Acid precipitated samples remained at room temperature for 20 min and were centrifuged at maximum speed in an International centrifuge for 20 min at 4° C. Absorbance was measured on a Hitachi 124 double beam spectrophotometer in a 1.0 cm light path, 1.0 ml quartz cuvette.
CHAPTER III

RESULTS

ENZYME CHARACTERIZATION

Existence of Alpha- and Beta-Galactosidases

Two lines of evidence indicated that hydrolysis of alpha- and beta-ONPG were the result of action by two distinct enzymes. (i) Cell lysates were electrophoresed as previously described, the gel columns were sectioned, halved, and assayed for galactosidase activity. Total activity was determined by summing the activities for all the sections of one gel tested. Table 4 illustrates the distribution of activities in the column sections, and indicates two, distinct, partially separated enzymes. (ii) N-ethyl maleimide (NEM) is an organic compound which ethylates free sulfhydryl groups. Cell lysates were treated with 100 mM NEM for 20 minutes at room temperature and assayed for galactosidase activity before and after treatment. Results in Table 5 indicated that alpha-galactosidase requires one or more sulfhydryl groups for activity, while beta-galactosidase does not.

Galactosidases are Soluble, Intra-cellular Enzymes

Cells were grown in 75 ml of TYE to an O.D. of 0.80. These were centrifuged and washed once in 20 ml of 0.001M potassium phosphate buffer (pH 6.8) plus 0.15% NaCl, and then were resuspended in 20 ml of the same. Fifty mg lysozyme were added and after protoplast formation was completed, the suspension was centrifuged. Protoplasts were re-
Table 4. Partial Separation of Alpha- and Beta-Galactoside Hydrolyzing Activity by Acrylamide Gel Electrophoresis

<table>
<thead>
<tr>
<th>Gel Section Number</th>
<th>Percent Hydrolysis of Alpha-ONPG**</th>
<th>Percent Hydrolysis of Beta-ONPG**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.0</td>
<td>5.3</td>
</tr>
<tr>
<td>2</td>
<td>0.0</td>
<td>0.1</td>
</tr>
<tr>
<td>3</td>
<td>1.3</td>
<td>1.0</td>
</tr>
<tr>
<td>4</td>
<td>78.4</td>
<td>4.8</td>
</tr>
<tr>
<td>5</td>
<td>18.8</td>
<td>61.1</td>
</tr>
<tr>
<td>6</td>
<td>0.3</td>
<td>27.6</td>
</tr>
<tr>
<td>7</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

*The gel column was sectioned at the interface of the stacking and resolving gels, and the latter was sectioned at 2.0, 2.5, 3.0, 3.5, and 4.0 cm from the gel interface. Sections were labeled as 1 through 7, were halved, and each section was assayed for alpha- and beta-ONPG hydrolyzing activity at 0.4 mM and 1.0 mM, respectively, at 60°C and pH 5.8.

**ONPG is the abbreviation for ortho-nitrophenyl galactoside.

Table 5. The Effect of N-Ethyl Maleimide Treatment of Alpha and Beta-Galactoside Hydrolysis

<table>
<thead>
<tr>
<th>Cell Lysate Treatment</th>
<th>Treated Lysate Activity x 100</th>
<th>Untreated Lysate Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alpha-ONPG** Hydrolysis</td>
<td>Beta-ONPG** Hydrolysis</td>
</tr>
<tr>
<td>Untreated</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>NEM** Treated</td>
<td>1</td>
<td>107</td>
</tr>
</tbody>
</table>

*ONPG is the abbreviation for ortho-nitrophenyl galactoside.

**Treated with 100 mM N-ethyl maleimide (NEM) for 20 minutes at room temperature prior to enzyme assays. Before and after treatment, lysates were assayed for alpha- and beta-ONPG hydrolyzing activity at 0.4 mM and 1.0 mM, respectively, in 0.1M potassium phosphate buffer (pH 5.6) at 60°C.
suspended in 20 ml distilled water, were sonicated and centrifuged. Samples were removed at each step for galactosidase assays. Results in Table 5 indicated that little enzyme activity is found in the growth medium, whole cell wash, or the supernatant fluid from the protoplast suspension. Both enzymes are released quantitatively upon lysis of the cells. Assays were 60 minutes at 60°C and pH 5.8 with alpha- and beta-ONPG at 0.4 mM and 1.0 mM, respectively.

**Constitutive Nature**

Cells were grown in TYE, were harvested, washed, and used as inocula for a series of minimal media with different carbohydrates as energy sources. Lactose and galactose have been shown to be inducers of beta-galactosidase in some other organisms (13, 22, 25). Melibose is a naturally occurring alpha-galactoside and glycerol was used for a non-inducing carbohydrate. Cells were grown in the minimal media, were harvested, lysed, and assayed for galactosidase activity. Results in Table 7 show that the carbohydrate has little effect on the enzyme activity of cells. However, the alpha-galactosidase activity of minimal-grown cells is significantly lower than cells grown in TYE. The reason for this difference is not clear, although other observations (data not shown) indicate that alpha-galactosidase levels are more susceptible to the glucose effect than are levels of beta-galactosidase within the cell.

**Enzyme Kinetics**

Kinetic data were obtained by assaying at pH 7.0 (0.1M potassium phosphate buffer) at 60°C for 30 minutes. Alpha-galactosidase activity
Table 6. Intracellular Location and Soluble Nature of Alpha- and Beta-Galactosidase

<table>
<thead>
<tr>
<th>Material Assayed</th>
<th>Sample Activity** Protoplast Activity x 100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alpha-Galactosidase</td>
</tr>
<tr>
<td>Supernatant Fluid From Culture</td>
<td>1.8</td>
</tr>
<tr>
<td>Whole Cell Wash</td>
<td>3.5</td>
</tr>
<tr>
<td>Whole Cells</td>
<td>96.4</td>
</tr>
<tr>
<td>Protoplasts</td>
<td>100.0</td>
</tr>
<tr>
<td>Supernatant Fluid From Protoplasts</td>
<td>4.0</td>
</tr>
<tr>
<td>Supernatant Fluid From Lysed Protoplasts</td>
<td>92.1</td>
</tr>
</tbody>
</table>

*Cells were grown in 2% Trypticase and 1% yeast extract, were harvested and washed once in 0.001M potassium phosphate buffer (pH 7.0). Cells were treated with lysozyme, were centrifuged, resuspended, lysed, and finally centrifuged.

**Preparations were assayed for activity at pH 5.8 and 60°C, and at alpha- and beta-ortho nitrophenyl galactoside concentrations of 0.4 mM and 1.0 mM, respectively.
Table 7. Determination of the Non-Inducible Nature of Alpha- and Beta-Galactosidase

<table>
<thead>
<tr>
<th>Growth Media</th>
<th>Enzyme Activity [(\frac{\text{u moles ONP}^*}{\text{min}}) (mg dry weight) \times 100]</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Alpha-Galactosidase</strong></td>
<td><strong>Beta-Galactosidase</strong></td>
</tr>
<tr>
<td>TYE**</td>
<td>1.08</td>
<td>3.28</td>
</tr>
<tr>
<td>Minimal Media + Glycerol</td>
<td>0.72</td>
<td>3.25</td>
</tr>
<tr>
<td>Minimal Media + Galactose</td>
<td>0.68</td>
<td>3.31</td>
</tr>
<tr>
<td>Minimal Media + Melibose</td>
<td>0.71</td>
<td>2.98</td>
</tr>
<tr>
<td>Minimal Media + Lactose</td>
<td>0.67</td>
<td>3.25</td>
</tr>
</tbody>
</table>

*ONP (ortho-nitrophenol).

**TYE (2% Trypticase plus 1% yeast extract).

(Note: Cells were grown on TYE and used as inocula for the minimal media with carbohydrates indicated. After growth, cells were harvested and lysed. Lysed preparations were assayed for alpha- and beta-ortho-nitrophenyl galactoside hydrolyzing activity at 0.4 mM and 1.0 mM, respectively, at 60°C and pH 5.8.)
was measured over a substrate range of 0.02 to 0.60 mM, while beta-galactosidase activity was tested over a range of 0.075 to 2.00 mM. Beta-galactosidase was plotted by the method of Hofstee (Figure 1) to determine maximum velocity \( V_{\text{max}} \) and Michaelis constant \( K_m \) for each enzyme. For alpha- and beta-galactosidase, the \( K_m \) values were 1.6 mM and 5.8 mM, respectively, and \( V_{\text{max}} \) values were 25.5 and 20.5 \( \frac{\mu \text{ moles}}{(\text{mg dry weight}) \times [\text{min} \times 10^2]} \), respectively.

Hydrogen Ion Concentration (pH) Optima

Using 0.1M citrate-phosphate buffer, cell lysates were tested for galactosidase activities over a wide pH range. Results shown in Figure 2 indicated that both enzymes have an optimum activity near pH 5.6. Assays were at 12 minutes at 60°C with 0.4 mM and 0.1 mM substrate concentrations for alpha- and beta-ONPG, respectively.

Temperature Optima

Cell lysates were assayed over a wide range of temperatures. Assays at pH 5.8 contained the same substrate concentrations as in the pH studies, and as seen in Figure 3, both galactosidases appear to have optimum temperatures between 65°C and 70°C. These values compare favorably with temperature optima for other thermophilic enzymes.

MEMBRANE STUDY

Effect of Calcium on Survival

Low DPG cells were suspended in 10 ml of 0.01M Tris buffer (pH 7.2) plus 0.004% glucose-6-phosphate, and in the same buffer plus 0.0125M CaCl₂, at a concentration of approximately \( 10^{-3} \)}
Figure 1. Kinetics of Alpha- and Beta-Galactosidase Activities

(Note: Crude cell lysates described in the "Methods and Materials" section were tested for hydrolysis of alpha-ortho-nitrophenyl galactoside (Δ—Δ) and beta-ortho-nitrophenyl galactoside (○—○) over substrate ranges of 0.02 to 0.60 mM and 0.075 to 2.00 mM, respectively. Assays were at 60°C in 0.1M potassium phosphate buffer (pH 7.0), and preparations were preheated for 5 minutes before addition of substrate. Maximum velocity ($V_{max}$) = y-axis intercept, and Michaelis constant ($K_m$) = y-axis intercept/x-axis intercept.)
Figure 2  Hydrogen Ion Concentration Optima of Galactosidase Activities

(Note: Crude cell lysates described in the "Methods and Materials" section were tested for hydrolysis of alpha-ortho-nitrophenyl galactoside (Δ) and beta-ortho-nitrophenyl galactoside (○) at 0.4 mM and 1.0 mM substrate concentrations, respectively. Assays were in 0.1M citrate-phosphate buffer at the indicated pH's and preparations were preheated for 5 minutes at 60°C before addition of substrate.)
Figure 3. Temperature Optima of Galactosidase Activities

(Note: Crude cell lysates described in the "Methods and Materials" section were tested for hydrolysis of alpha-ortho-nitrophenyl galactoside (○) and beta-ortho-nitrophenyl galactoside (▲) at 0.4 mM and 1.0 mM substrate concentrations, respectively. Assays were in 0.1M potassium phosphate buffer (pH 7.0), and preparations were preheated for 5 minutes at the respective temperatures before addition of substrate.)
cells per milliliter. At times indicated, samples were removed and plated. Results in Figure 4 indicated rapid loss of viability in the buffer without calcium within 10 minutes, while cells in the buffer with calcium show an initial increase followed by a slight decrease over an 80-minute period. The results indicated that calcium markedly enhances the survival of *B. stearothermophilus* in Tris buffer at 60°C. Survival in 0.001M phosphate buffer (pH 7.2) also requires calcium. However, survival in the phosphate buffer plus calcium was slightly less than in the Tris buffer plus calcium.

If the cells were suspended in 0.001M potassium phosphate buffer at pH 6.8 (K-PO₄ buffer) at cell densities comparable to those used following the release of cellular constituents (10¹⁰ to 10¹¹ cells per milliliter), they survived at pH 7.2 in the presence of calcium. If the pH was lowered to 6.8, the cells did not survive, whether or not calcium was present (Figure 5). As the O.D. measurements at 600 nm indicated, however, calcium did appear to stabilize cells against lysis.

**Effect of Calcium on Release of Cellular Constituents**

A. **Release of protein.** *Bacillus stearothermophilus* showed rapid release of protein from whole cells in K-PO₄ buffer at 60°C, but addition of calcium and glucose significantly reduced the amount of protein released. Cells in K-PO₄ buffer released 67% of the total cellular protein in 120 minutes, while those in the buffer plus calcium and glucose released only 12% (Figure 6). The addition of glucose alone provided little protection for the cells against loss of protein, while
Figure 4. Effect of Calcium on Survival of Bacillus stearothermophilus

(Note: Growing cultures were diluted $10^{-4}$ at mid-log phase into 0.01M Tris buffer (pH 7.2) plus 0.004% glucose-6-phosphate (○) or the same buffer mixture plus 0.0125M CaCl$_2$ (●). Both buffers were preheated to 60°C. Samples were removed and plated at the times indicated.)
Figure 5. Effect of Calcium on Changes in Viability of *Bacillus stearothermophilus* and O.D. at pH 6.8

(Note: Mid-log phase cells were resuspended (10^{10} to 10^{11} cells per milliliter) in potassium phosphate buffer at pH 6.8 (●) and in potassium phosphate buffer at pH 6.8 plus 0.0125M CaCl₂ plus 0.01% glucose (○), and the O.D. at 600 nm (-----) and viable cell count (-----) checked at indicated times while heating at 60°C.)
Figure 6. Factors Affecting Release of Protein from \textit{Bacillus stearothermophilus} Cells Heated at 60°C

(Note: Mid-log phase cells were resuspended in 10^{10} to 10^{11} cells per milliliter in the buffer indicated. Ca^{++} and Mg^{++} were both at 0.0125M, and glucose was at a concentration of 0.01%. Cells were heated at 60°C, and supernatants which had been collected by centrifugation were assayed for protein content. Total protein was determined by assaying unheated dilutions of each buffered cell suspension.)
calcium appeared to be as effective as calcium plus glucose (Figure 6). Another divalent cation, magnesium (in a buffer of K-PO₄ plus 0.0125M MgCl₂ plus 0.01% glucose), also protected the cells from protein loss (Figure 6). Magnesium did not appear to be as effective as calcium at equivalent concentrations, and after 120 minutes at 60°C, the cells in K-PO₄ buffer plus magnesium released 19% of the total cellular protein, compared to the K-PO₄ buffer plus calcium, in which the cells had released only 12% (Figure 6).

The effect of varying concentrations of calcium on the release of protein from cells of *B. stearothermophilus* is shown in Figure 7. In the absence of glucose, calcium appeared to give increasing protection against loss of protein with increasing calcium concentrations between 10⁻⁴ and 10⁻² M. It should be noted that, while calcium concentrations of up to 10⁻² M did not interfere with the Lowry determination of protein, higher concentrations did interfere. Thus, the protective effects of calcium above 10⁻² M cannot be quantified by protein release.

Further evidence that calcium rather than glucose was the component responsible for the majority of this protective effect is seen in Figure 8. Comparing protein released from cells in K-PO₄ buffer plus 0.01, 0.10, and 1.00% glucose indicated little increase in resistance to protein release with increasing glucose concentrations. As seen in Figure 8, the 0.01% glucose concentration gives about 35% protection after 80 minutes at 60°C by reducing the amount of total cellular protein released from 35% to 21%. The data in Figure 6, however, suggest that this difference remains constant as the heating time increases. Thus, in Figure 6, the difference between release of protein
Figure 7. The Effect of Varying Concentrations of Calcium on Protein Release from Mid-Log *Bacillus stearothermophilus* Cells

(Note: Mid-log phase cells were resuspended in the above buffers at 10^{10} to 10^{11} cells per milliliter, and were heated at 60°C. Collected supernatants were assayed for protein content, and total protein was determined by assaying dilutions of unheated suspension for each buffer.)
Figure 8. The Effect of Varying Glucose Concentrations on Release of Protein from Heated Whole Cells of *Bacillus stearothermophilus*

(Note: Mid-log phase cells were harvested and resuspended (10^{10} to 10^{11} cells per milliliter) in potassium phosphate buffer at pH 6.8 plus the glucose concentrations indicated. Cells were heated for 80 minutes at 60°C, and supernatant which had been collected by centrifugation was assayed for protein content. Total protein was determined by assaying dilutions of unheated preparations.)
from K-PO₄ buffer and from K-PO₄ buffer plus glucose is 6% total cellular protein after 120 minutes. However, the percent protection (protein released in K-PO₄ buffer plus glucose divided by the amount of protein released in K-PO₄ buffer alone) decreased, and is 17% after 15 minutes at 60°C, while it is only 13% after 120 minutes. Thus, the protection added by glucose in the K-PO₄ buffer plus calcium plus glucose appears to be minimal.

B. Release of galactosidases. Cell leakage can be measured by following the release of galactosidases. Supernatant fluid assays (Figure 9) indicated rapid release of alpha- and beta-galactosidase from cells heated at 60°C in K-PO₄ buffer, while cells in K-PO₄ buffer plus 0.0125M CaCl₂ plus 0.01% glucose showed a much reduced rate of release. Little release could be detected in any of the cells after 10 minutes, but after 40 minutes at 60°C, cells in K-PO₄ buffer had released 58 and 66% of alpha- and beta-galactosidase, respectively. Cells in the K-PO₄ buffer plus calcium and glucose, however, released only 9% of alpha-galactosidase and 13% beta-galactosidase after 120 minutes at 60°C. Since both enzymes appeared to be released at about the same rate, subsequent work was restricted to beta-galactosidase.

C. Beta-galactosidase activity of whole cells. The beta-galactosidase activity of whole cells of *B. stearothermophilus* normally was about 60% that of the corresponding lysate activity. Work with mesophilic systems has yielded similar results. It is generally accepted that this occurs because transport is the slow step in hydrolysis of galactosides by whole cells. If beta-galactosidase were released
Figure 9. The Effect of Calcium on the Release of Galactosidases from Heated Whole Cells of *Bacillus stearothermophilus*

(Note: Mid-log phase cells were resuspended in 0.001M potassium phosphate buffer at pH 6.8 alone (- - -) and in 0.001M potassium phosphate buffer (pH 6.8) plus 0.0125M CaCl₂ plus 0.01% Glucose (-----), were heated, centrifuged, and the supernatants were tested for beta-galactosidase (○) and alpha-galactosidase (△) activity.)
selectively, but the integrity of the membrane maintained, then one would expect the increase in whole cell activity to correspond to the amount of enzyme released plus the remaining whole cell activity. The results in Figure 10 indicated that the activity of the whole cells after 40 minutes at 60°C was equal to that of the lysates, while only 65% of the activity could be accounted for in the supernatant at that time. This implied a breakdown in the permeability barrier to substrate penetration. Calcium plus glucose, on the other hand, protected against increases in the whole cell activity (Figures 9 and 10).

D. Release of beta-galactosidase. Calcium concentrations of $10^{-1}$ M in the K-PO$_4$ buffer ($5 \times 10^{-2}$ M in the enzyme assay system) did not interfere with the assay of beta-galactosidase, contrasting to the interference with the Lowry determination of protein mentioned earlier. Concentrations for $10^{-3}$ to $10^{-1}$ M gave increasing protection against leakage of beta-galactosidase from heated whole cells (Figure 11). Furthermore, $10^{-1}$ M calcium gave complete protection and prevented leakage of detectable amounts of beta-galactosidase from heated whole cells.

E. Release of nucleotides. As shown in Figure 12, the amount of ribose, assumed to be RNA (5), released in the presence of calcium after 60 minutes at 60°C was only about 25% the amount released from cells in the absence of calcium. When supernatant fluids were scanned from 240 to 200 nm (260/280 ratios were usually 3/1, although less for the acid soluble fraction from K-PO$_4$ buffer plus calcium heated cells) two important observations were noted: (i) the amount of 260 nm-absorbing
Figure 10. The Effect of Calcium on Beta-Galactosidase Activity of Heated Whole Cells of *Bacillus stearothermophilus*.

(Note: Mid-log phase cells were resuspended in 0.001M potassium phosphate buffer (pH 6.8) (○) and in 0.001M potassium phosphate buffer (pH 6.8) plus 0.0125M CaCl$_2$ plus 0.01% glucose (Δ), were heated, removed to an ice bath, and were tested for beta-galactosidase activity.)
Figure 11. Effect of Varying Calcium Concentrations on Release of Beta-Galactosidase from Heated Whole Cells of *Bacillus stearothermophilus*

(Note: Cells were harvested at mid-log phase and resuspended in the indicated buffer at 10^10 to 10^11 cells per milliliter. After heating at 60°C, the supernatants which had been collected by centrifugation were assayed for beta-galactosidase activity. Total activity was determined by assaying whole cell lysates.)
Figure 12. The Effect of Calcium on the Release of Ribose from *Bacillus stearothermophilus* Cells Heated at 60°C

(Note: Mid-log Phase Cells were resuspended at $10^{10}$ to $10^{11}$ cells per milliliter in the buffers indicated. After heating at 60°C, the supernatants which had been collected by centrifugation were assayed for ribose by the orcinol reaction.)
material released in the presence of calcium (Figure 13) is only about
30% as much as that released when calcium is excluded from the heating
buffer system; and (ii) essentially all the material released in the
presence of calcium is acid-soluble, while only about 25% of the material
released in the absence of calcium is acid-soluble. This indicated that
nucleotides released in the presence of calcium are primarily oligo-
nucleotides, while two-thirds of the nucleotides released in the absence
of calcium are larger molecular weight polynucleotides. Results in
Figure 13 indicate that the acid-soluble nucleotides released in the
absence of calcium are less than those released in its presence.
However, oligonucleotides often co-precipitate with protein during
acid-precipitation (50), and the results in Figure 6 indicate the pre-
sence of a substantial amount of protein released in K-PO₄ buffer without
calcium. Therefore, this difference is probably caused by the co-
precipitation of some of the oligonucleotides with the protein in the
sample.

These combined results indicated that cells heated in K-PO₄
buffer at 60°C rapidly lyse and show non-specific release of all
intracellular components. Calcium ions protected against such lysis.

Effect of Anaerobiosis on Survival

Dr. Card (private communication) has shown that if mid-log
phase cells are held anaerobically at 60°C, there is conversion of
PG to DPG, and cessation of PG synthesis. Steady-state for this enzy-
matic conversion is reached after 15 to 20 minutes of anaerobiosis. If,
however, aerobic conditions are resumed in the presence of an energy

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Figure 13. The Effect of Calcium on the Release of 260 nm-Absorbing Material from *Bacillus stercothermophilus* Whole Cells

(Note: Mid-log phase cells were resuspended at $10^{10}$ to $10^{11}$ cells per milliliter and were heated in 0.001M potassium phosphate buffer (pH 6.8) alone (●) and in potassium phosphate buffer plus 0.0125M CaCl$_2$ plus 0.01% glucose (▲) at 60°C. Supernatants were collected and diluted 1:2 with potassium phosphate buffer (---) or were precipitated with potassium phosphate buffer plus 14% trichloracetic acid (-----). The latter were centrifuged after 20 minutes at room temperature. O.D. units were calculated by multiplying the actual O.D. reading by the dilution factor required to obtain an O.D. at 260 nm of 0.2 to 2.0.)
source, DPG turnover and PG synthesis resumes, and the PG/DPG ratio increases and returns to the ratio typical of mid-log phase cells. High DPG cells (those held anaerobically) show no enhanced survival compared to low DPG cells in Tris buffer (pH 7.2) plus glucose-6-phosphate at cell densities of $10^4$ cells per milliliter (data not shown). The probable reason for this phenomenon is that aerobic conditions prevail at these cell densities and PG synthesis and DPG turnover occur. Even at these cell densities, however, high DPG cells show enhanced survival compared to low DPG cells when this buffer is supplemented with 0.005% Trypticase (Figure 14). During a 20-minute period in Tris buffer (pH 7.2) plus glucose-6-phosphate plus 0.005% Trypticase at 60°C, the high DPG cells were observed to increase slightly, while the low DPG cells showed a hundred-fold loss in the same period. In Trypticase concentrations of 0.01% of above, both populations of cells survive equally well, while at Trypticase concentrations of 0.0002% or less, neither population survived without the addition of calcium (data not shown).

It is thus apparent that even under aerobic conditions (which prevail for the buffer condition) described in the legend of Figure 14, in which the initial low PG/DPG ratio is not maintained, the high DPG cells show an enhanced survival rate compared to low DPG cells.

**Effect of Anaerobiosis on Release of Cellular Constituents**

A. **Release of protein.** Release of protein from low DPG cells at 60°C in K-PO₄ buffer has already been demonstrated. Figure 15 illustrated the release of protein from low DPG cells and high DPG

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Figure 14. The Effect of Anaerobiosis on Survival of *Bacillus stearothermophilus* Cells

(Note: Growing cultures at mid-log phase ( ), low diphosphatidyl (DPG), or those held for 20 minutes anaerobically ( ), high DPG, were diluted 10⁻⁴ into 0.01M Tris buffer (pH 7.2) plus 0.004% glucose-6-phosphate plus 0.005% Trypticase, which had been preheated at 60°C. Samples were removed and plated at times indicated.)
Figure 15. The Effect of Anaerobiosis on Release of Protein from Bacillus stearothermophilus Cells

(Note: Cells were harvested at mid-log (○), low diphosphatidyl (DPG), or after 30 minutes anaerobiosis (●), high DPG, and were resuspended in 0.001M potassium phosphate buffer (pH 6.8) at $10^9$ to $10^{11}$ cells per milliliter. After heating at 60°C, the supernatants which had been collected by centrifugation were assayed for protein content. Total protein was determined by assaying unheated preparations.)
cells, the latter obtained by incubation for 30 minutes anaerobically prior to harvesting. The results indicated that there was a marked reduction in release from the high DPG cells. After 120 minutes at 60°C, low DPG cells showed release of 66% of the total cellular protein, while high DPG cells released only 21% after the same incubation time. The addition of calcium to high DPG cells further reduces this release as seen in Figure 16. High DPG cells without calcium released 21% total cellular protein, compared to only 7% after 120 minutes at 60°C when calcium was included in the K-PO₄ buffer.

B. Release of beta-galactosidase. Portions of a cell population were held for increasing times anaerobically prior to harvesting, and the amount of beta-galactosidase released upon heating showed a corresponding decrease. Figure 17 illustrates the effect of 10 and 20 minutes of anaerobiosis on the release of beta-galactosidase from these cells compared to those harvested at mid-log phase (low DPG) with no anaerobic incubation. The first 10 minutes of anaerobiosis resulted in a marked reduction in the rate of release, while 20 minutes of anaerobiosis increased the cells' resistance to leakage only slightly beyond that of the 10 minute cells.

C. Release of nucleotides. Release of RNA from anaerobic cells was greatly reduced compared to those harvested at mid-log phase as is shown in Figure 18. After 60 minutes at 60°C in K-PO₄ buffer, only 30% as much RNA had been released from the anaerobic cells as from those not incubated anaerobically prior to harvesting. It is apparent that anaerobiosis increases the stability of cells at 60°C and greatly

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Figure 16. The Effect of Calcium on the Release of Protein from High DPG* cells of *Bacillus stearothermophilus*

(Note: Cells were held anaerobically for 20 minutes, high diphosphatidyl, were harvested and resuspended at $10^{10}$ to $10^{11}$ cells per milliliter in potassium phosphate buffer (pH 6.8, 0.001M) alone (○), and in potassium phosphate buffer plus 0.001M CaCl$_2$ (●). After heating, supernatants which had been collected by centrifugation were assayed for protein. Total protein was determined by assaying dilutions of unheated preparations.)

*DPG (diphosphatidyl glycerol)
Figure 17. The Effect of Varying Anaerobic Periods on the Release of Beta-Galactosidase from *Bacillus stearothermophilus* Cells

(Note: Cells were harvested at mid-log phase or were held for 10 and 20 minutes anaerobically prior to harvesting. Cells were resuspended in potassium phosphate buffer (0.001M, pH 6.8) at 10^10 to 10^11 cells per milliliter, were heated, and the supernatants which had been collected by centrifugation were assayed for beta-galactosidase activity. Total beta-galactosidase was determined by assaying lysed whole cell preparations.)
Figure 18. The Effect of Anaerobiosis on the Release of Ribose from Bacillus stearothermophilus Cells Heated at 60°C

(Note: Cells were harvested at mid-log phase (○), low diphosphatidyl (DPG), or after 30 minutes of anaerobiosis (●), high DPG, and were resuspended in potassium phosphate buffer (0.001M, pH 6.8) at 10^{10} to 10^{11} cells per milliliter. After heating at 60°C, the supernatants which had been collected by centrifugation were assayed for ribose content by the orcinol reaction.)
reduces the amount of lysis which occurs in low DPG cells without calcium.

Effect of Monovalent Cations on Protection by Calcium

Monovalent cations appeared to compete with calcium for some binding sites on an apparent non-specific basis. A comparison of the effects of Na\(^+\), K\(^+\), and NH\(_4^+\) on the protection by calcium is shown in Figure 19. The addition of 5 \times 10^{-2} M concentrations of these ions (as chloride salts) all reduce the protection by 5 \times 10^{-3} M Ca\(^{2+}\) to about the same degree.
Figure 19. The Effect of Monovalent Cations on the Protection by Calcium of *Bacillus stearothermophilus* Cells

(Notes: Mid-log phase cells were harvested and resuspended at $10^{10}$ to $10^{11}$ cells per milliliter in the buffers indicated, with the Ca$^{2+}$ concentration at $5 \times 10^{-3}$ M, and Na$^+$, NH$_4^+$, and K$^+$ concentrations all at $5 \times 10^{-2}$ M. Cells were heated at 60°C for 60 minutes, and the supernatants which had been collected by centrifugation were assayed for beta-galactosidase. Total beta-galactosidase was determined by assaying whole cell lysates.)
CHAPTER IV

DISCUSSION

Originally, it was determined that *B. stearothermophilus* possessed enzymes which hydrolyzed alpha- and beta-galactosides. It was hoped that a greater insight into membrane organization would be gained by studying galactoside transport. The beta-galactoside transport system has been worked on and was well characterized in both *Staphylococcus aureus* (14, 20, 21, 23) and *E. coli* (26, 27). It had been shown to be a phosphoenol pyruvate- (PEP) requiring system in both organisms (20, 23, 26). Kundig and Roseman (26, 27), working with similar PEP transport systems in *E. coli* for glucose, fructose, and mannose, have shown that phosphorylation of the sugar has a specific requirement for PG. The work with *B. stearothermophilus* was intended to determine the following: (i) whether or not PEP galactoside transport was similarly operative in *B. stearothermophilus*; (ii) a specific phospholipid requirement for transport in this organism; and (iii) the effect of varying membrane phospholipid composition on transport of galactosides.

Initial studies in this direction did not appear fruitful, and indicated a need for several preliminary steps. Firstly, it seemed necessary to characterize the galactosidases. Secondly, it appeared that membrane stability was lost during the enzyme assays; therefore, it was felt paramount to try to determine the requirements for membrane stabilization under these conditions. Without such determinations,
it would be impossible to study the galactoside transport systems. The latter step, however, appeared to represent an entire study in itself, and to possess the potential of giving unique insight into the problem of thermophily and membrane organization and function.

The characterization of alpha- and beta-galactosidases has yielded no results leading to new insights into thermophily or membrane physiology. As was noted, the temperature optima for both enzymes (60 to 70°C) were comparable with those obtained by other workers with a variety of enzymes from *B. stearothermophilus* (4, 33, 36, 37, 41, 42, 60). The only surprising observation was that both galactosidases were constitutive and not inducible. This is not, however, an attribute universal to thermophiles, since Ulrich (60) has reported that beta-galactosidase was inducible in a thermophile resembling *Thermus aquaticus*. Also, the alpha-amylase of Manning and Campbell (63, 64) was partially inducible. In subsequent studies on membrane stability, however, the applicability of using the galactosidases as indicators of cell lysis was dependent on three aspects of the nature of these enzymes: (i) *Bacillus stearothermophilus* possesses two distinct galactosidases (alpha and beta); (ii) both galactosidases are intracellular enzymes; and (iii) both enzymes are soluble. These two enzymes, therefore, were useful yardsticks of cell lysis and/or leakage of intracellular components, as well as showing that release of intracellular protein was not selective.

Ljunger (30) had previously demonstrated the effect of calcium on thermal viability of *B. stearothermophilus*, and subsequent work confirmed some of his conclusions. Results indicated survival of
cells in 0.01M Tris buffer (pH 7.2) plus glucose-6-phosphate, and in 0.001M K-PO₄ buffer plus glucose if calcium was included, but none in its absence. With higher cell densities used in subsequent release studies, similar survival was noted at pH 7.2. If the pH was lowered to 6.8, however, cells did not survive with or without calcium, although O.D. measurements indicated that calcium protected against extensive lysis. With the latter buffer system, results have shown that the absence of calcium results in lysis of the cells when incubated at 60°C in K-PO₄ buffer. Lysis was measured by O.D. changes at 600 nm and by the release of alpha- and beta-galactosidase, 260 nm-absorbing material, protein, and RNA. In the absence of calcium, virtually all the soluble enzymes (using both galactosidases as indicators) and a majority of the total cellular protein (67%) are released after 120 minutes at 60°C. Calcium significantly diminished release of these components and showed increasing protection with increasing concentration. Nucleotides (260 nm-absorbing material) released in the presence of calcium were primarily oligonucleotides, while four times as much material was released in its absence, two-thirds of which were polynucleotides.

The effect of calcium, therefore, was to increase the stability of the cell membrane. These particular results, however, give little insight as to whether this was a direct effect on the membrane or on some other cellular constituent which in turn stabilized the membrane.

Dr. Card (11) has shown that the phospholipids of _B. stearothermophilus_ are associated primarily with the cytoplasmic membrane. The effect of anaerobiosis on changes in phospholipid composition then are not subject to the criticism which can be leveled at the work with cal-
That is, the effect of calcium has not been shown to be a membrane phenomenon, but conversion of two molecules of PG to DPG is solely associated with the membrane. Results have shown that high DPG cells survive better than low DPG cells in 0.01M Tris buffer (pH 7.2) plus glucose-1-phosphate plus 0.005% Trypticase, although experimental data does not explain the Trypticase requirement at this time. Rates of lysis observed by release of beta-galactosidase, protein, and RNA also indicated that high DPG cells have a much more stable membrane than low DPG cells, and that the effect of increasing DPG concentrations resulted in further increases in stability.

Any explanations for these effects must be compatible with the present concept of membrane structure. Singer (55, 56) and others (19, 38) have indicated that most of the membrane protein (approximately 70%) is apparently an integral part of the membrane, and that it resides within a lipid layer which constitutes most of the membrane lipid (39, 55). One the basis of this model, it appears that two explanations may account for the observed increase in membrane stability following increases in DPG concentrations.

The first hypothesis is that conversion of two PGs to DPG may link protein units together within the membrane. The result would be a membrane in which protein was extensively cross-linked with other protein in the following manner. Symbols and the molecules they represent are depicted in Figure 20. Figure 21 shows a schematic view of a membrane cross-section before and after conversion of two molecules of PG to DPG, and Figure 22 is a representation of the top view of the membrane.
Figure 20. Structures and Schematic Representations of Phosphatidyl Glycerol (PG) and Diphosphatidyl Glycerol (DPG) and Linkage with Protein Units

(Note: the circles represent glycerol moieties and wavy lines the hydrocarbon chains of esterified fatty acids.)

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Figure 21. Schematic Representation of a Membrane Cross-Section Before and After Conversion of Two Molecules of Phosphatidyl Glycerol to Diphosphatidyl Glycerol

Figure 22. Schematic Representation of the Top View of a Membrane Before and After Conversion of Two Molecules of Phosphatidyl Glycerol to Diphosphatidyl Glycerol
The other explanation is that bilayers of DPG are simply more stable (have a higher surface tension) than bilayers of PG. Thus, bilayer A (Figure 23) may be less stable than bilayer B.

Figure 23. Schematic Representations of Bilayers of Phosphatidyl Glycerol (Bilayer A) and Diphosphatidyl Glycerol (Bilayer B)

At present, there is no way of distinguishing between the two alternatives, although the surface tension of lipid bilayers of PG and DPG could be determined and compared. Such results would probably, but not necessarily, support one hypothesis or another.

There are at least four other explanations which might be proposed to account for the effect of calcium: (i) calcium stimulates the enzyme responsible for conversion of two PG molecules to one molecule of DPG; (ii) calcium inhibits autolysins, e.g., proteases, nucleases, and/or muramidases; (iii) calcium forms ionic bridges between membrane lipid moieties and stabilizes the membrane; and (iv) calcium forms ionic bridges between membrane protein moieties and stabilizes the membrane.

The first explanation is dependent upon evidence that DPG
synthesis is stimulated by calcium. Dr. Card (personal communication) has indicated that calcium has no apparent effect on conversion of PG to DPG. The second explanation has not been tested either in this laboratory or, apparently, elsewhere. Indirect evidence, however, indicates that it is probably not the explanation. One must note that two divalent cations, calcium and magnesium, both stabilize cells and that three monovalent cations, sodium, ammonium, and potassium, all reduce the protection by calcium to about the same degree. To contend that one type of autolysin (and more likely all three) would be inhibited by two different divalent cations and activated (or divalent cation inhibition blocked) by three different monovalent cations, is not consistent with the specificity evidenced for enzymes.

The third explanation, on the other hand, is based upon some indirect experimental evidence. It has been shown that conversion of two molecules of PG to one of DPG increases membrane stability. Since PG possesses negatively charged phosphate groups, it is conceivable that calcium could form ionic bridges between these phosphate groups and mimic the effect of formation of DPG. Figure 24 portrays the formations of these bridges.

![Figure 24. Schematic Representation of the Formation of Ionic Bridges Between Membrane Lipid Moieties by Calcium to Mimic the Effect of Formation of Diphosphatidyl Glycerol](image-url)
Since conversion of PG to DPG is always in equilibrium (i.e., some PG always remains), and since DPG also possesses charged phosphate groups, this would be compatible with the observation that calcium increases the stability of high DPG cells. This observation, however, is also compatible with the fourth explanation. This prediction concerning the effect of calcium on membrane lipids has some other experimental support. Rubalcava et al. (49) have found that divalent cations (i.e., calcium and magnesium) increased the staining both of lipid micelles of dipalmitoylphosphatidylcholine and of microsomal membranes by 1-anilinonaphthalene-8-sulfonic acid. This stain has been shown to associate with the hydrophobic region of lipids (38,58). The effect of this increased staining was attributed to the shrinkage of the lipid bilayers resulting from the ionic bonds formed between the divalent cation and the polar ends of the phospholipids (58). Such results implicate the membrane lipids but do not eliminate the possible role(s) of protein for biological membrane systems.

Thus, the last explanation appears to be in limbo. It is, in fact, impossible to say at this point that it is lipid and not protein to which the calcium binds, or to exclude the possibility that both are involved. But perhaps more importantly, it appears that there are no available techniques which would allow one to distinguish among these explanations.

It was Ljunger's contention (28, 29, 30) that the effect of calcium was to stabilize cellular protein against heat denaturation. On the basis of previous research with thermophilic enzymes (3, 60), his conclusion did not seem warranted. Evidence indicated that increases
in the amount of DPG in the membrane also increased thermal viability of the organism in the absence of calcium. Both calcium and high DPG were subsequently shown to increase the stability of the cells by directly affecting the cells membrane. Organization of multi-enzyme systems and their effective activity and control in a living organism is a state more easily effected by subtle changes than is membrane stability. It is thus apparent that events leading to the loss of stability would first result in loss of organization. In the light of the evidence presented, and in the absence of any evidence supporting Ljunger's hypothesis, it is postulated that loss of thermal viability cannot be attributed to massive stabilization of cellular protein against heat denaturation, but rather to membrane disorganization. Furthermore, both calcium and high DPG stabilize cells against thermal death by directly increasing membrane stability and reducing the rate of metabolic disorganization under non-growing conditions.
CHAPTER V

SUMMARY

Results indicated that divalent cations (Ca\textsuperscript{++} and Mg\textsuperscript{++}) and high concentrations of DPG increase the stability of \textit{B. stearothermophilus} cells by direct interaction with the cell membrane.

Stability was measured by following the release of cellular constituents from whole cells of \textit{B. stearothermophilus} when heated at 60°C. Cells with low DPG content, when heated in the absence of divalent cations showed rapid release of alpha- and beta-galactosidase (previously unreported for this organism), protein, ribose, and 260 nm-absorbing material. Addition of divalent cations or anaerobic incubation of cells prior to harvesting (resulting in high DPG concentrations) significantly reduced the amount of cellular constituents released during heating. In addition, both calcium cations and high DPG cells were shown to have increased rates of survival at 60°C compared to low DPG cells without calcium.

The significance of these observations to the concepts about thermophily and membranes are discussed.
LITERATURE CITED


